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Determination of Adenosine Phosphates in Whole Blood by Capillary Zone Electrophoresis[1])

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Summary: The pool of chemical energy in an organism represented by high-energy compounds can be assessed by means of adenosine triphosphate (ATP) determination in whole blood and tissues. The elegant manner for the determination of adenosine phosphates (ATP, ADP, AMP) in a single assay is offered by the technique of capillary zone electrophoresis. For this purpose, the BioFocus 3000 Capillary Electrophoresis System (BIO-RAD Laboratories, Inc., Hercules, CA, USA) was used. For the construction of calibration curves, pure preparations of ATP, ADP and AMP were analyzed. The method was used for adenosine phosphates determination in the umbilical blood samples from physiological and immature newborns. Capillary zone electrophoresis enables a specific and simultaneous determination of adenosine phosphates and, thus, monitoring of unusual metabolic situations.

Introduction

The determination of adenosine phosphates concentrations, especially of adenosine triphosphate (ATP), in whole blood and various tissues can be used for estimating the state of the pool of chemical energy in an organism. ATP represents the energetic currency of the cell, and is a measure of exergonic biochemical processes. Hypoxaemia influences the endothelial cells – interface between blood and tissues – and the decrease of ATP content alters their functions and can seriously impair organs (1).

In this study, a simplified approach using capillary electrophoresis for adenosine phosphate determination in whole blood within a single analysis was taken. The method is considered helpful for evaluation of the anoxic period during the birth period and of the oxygen supply to organs of newborns and preterm babies during the first days of life (2). Capillary electrophoresis for the separation of purine bases and nucleosides in human cord plasma was used by *Grune* et al. (3), however, rather with regard to other purine compounds than nucleotides. *Dawson* et al. developed a capillary electrophoresis method using an uncoated capillary to resolve potential impurities in a phosphonate analogue of adenosine triphosphate (4).

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Experimental

Apparatus

A BioFocus 3000 Capillary Electrophoresis System (BIO-RAD Laboratories, Inc., Hercules, CA, USA) was used for analyses. The fused silica capillaries as capillary cartridges (24 cm × 25 µm, coated) are available from BIO-RAD Laboratories. The instrument was run according to the manual of the producer (5). Many valuable and important notions about the capillary electrophoresis techniques were obtained from the monography of *Landers* and coauthors (6).

Chemicals

Adenosine 5'-triphosphate disodium salt × 3 H_2O and adenosine 5'-monophosphate disodium salt × 6 H_2O were obtained from Boehringer Mannheim GmbH, Germany. Adenosine 5'-diphosphate sodium salt was obtained from SIGMA Chemical Co., St. Louis, Mo, USA, perchloric acid 70% (= 700 g/kg) was purchased from Carlo Erba, Milano, Italy, triethanolamine hydrochloride and potassium carbonate anhydrous from FLUKA, Buchs, Switzerland, 0.23 mol/l borate buffer pH 7.8 was modified from the original 0.3 mol/l borate buffer pH 8.5 provided by BIO-RAD Laboratories, CA, USA.

Sample pretreatment

The samples of heparinized blood were deproteinized immediately after being taken, with 1:20 diluted perchloric acid (700 g/kg) in a 1 : 1 ratio. After centrifuging for 10 min at 4500 *g*, four parts of a supernatant were neutralized (in an icebath) with one part of a 1 mol/l solution of triethanolamine hydrochloride and 1.3 mol/l potassium carbonate under simultaneous precipitation of perchloric acid. The mixture of 50 µl supernatant and 5 µl operational 0.23 mol/l borate buffer pH 7.8 was analyzed. The same procedure was used for various concentrations of pure preparations of adenosine phosphates taken as a standard sample set.

Analysis conditions

Buffer, samples, and all flushing solutions were used after filtration through a 0.45 µm filter (Micro Prep-Disc BIO-RAD) and deaera-

tion under reduced pressure (water aspiration pump). For optimal performance, the capillaries were preconditioned with 0.1 mol/l NaOH for 2 min, with deionized water for 2 min and, finally, 3 min with operational borate buffer 0.23 mol/l, pH 7.8 before the first use. Between runs, the capillaries were purged for 1 min with deionized water, 1 min with 0.1 mol/l NaOH, 1 min with deionized water and 2 min with run buffer. The separations were run with the direction of electrode polarization ⊖ → ⊕, at 20 °C capillary cartridge and carousel temperature, constant voltage 20 kV (3 min) or 10 kV (6 min) respectively. The samples were injected into the capillary with a pressure of 5 psi (3.5 × 10^3 kg/m^2) 4 seconds (pressure time inject constant 20), detection at 260 nm.

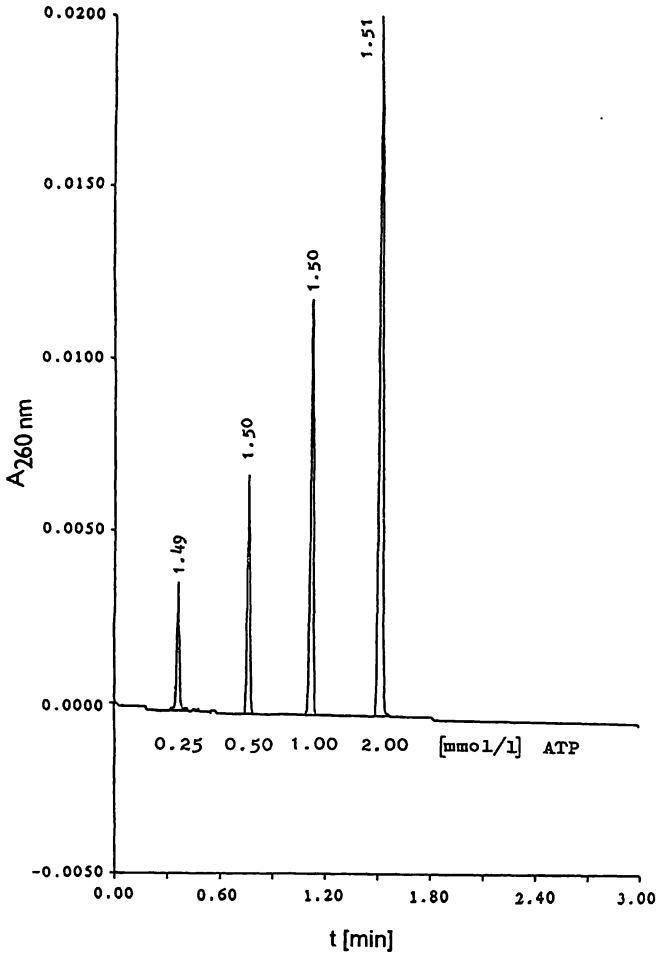
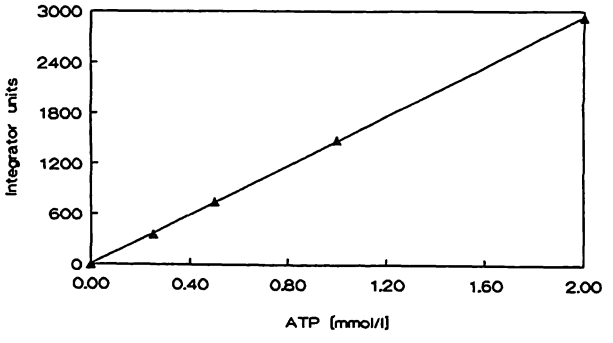


Fig. 1a The analyses of ATP standard solutions with concentrations between 0.25 and 2.00 mmol/l. Separation conditions: coated silica capillary (25 µm I.D. × 24 cm, 19.4 cm to the window). Operational borate buffer 0.23 mol/l, pH 7.8. Injection of sample 20 psi (14 × 10^3 kg/m^2) × s, 20 °C, 20 kV, polarity ⊖ → ⊕, detection wavelength 260 nm.



No.	ATP (mmol/l)	Integrator units
1	0.00	0.0
2	0.25	362.0
3	0.50	746.4
4	1.00	1485.8
5	2.00	2923.2

Fig. 1b Calibration graph of ATP. Data from analyses see figure 1a.

Results

The results for ATP standard solutions with concentrations between 0.25 and 2.0 mmol/l and of the concentration ranges of adenosine nucleotides (ATP, ADP, AMP) expected in whole cord blood deproteinates are shown in figures 1a and 2a together with calibration graphs (figs 1b and 2b). The linearity and reproducibility of migration times is evident. The results in figure 2a show ATP, carrying near the neutral pH four net negative charges as the first nucleotide passing the detector. Adenosine diphosphate with three, and AMP with two, net negative charges sucessively pass the detector through the coated capillary with the direction of electrode polarization ⊖ → ⊕. Table 1 shows the migration times (mean ± SD) and apparent electrophoretic mobilities of adenosine phosphates separated by capillary electrophoresis under the conditions described in figure 2a. The reproducibility of the migration times and peak areas were investigated for a single blood sample analyzed several times (tab. 2). The identity of nucleo-

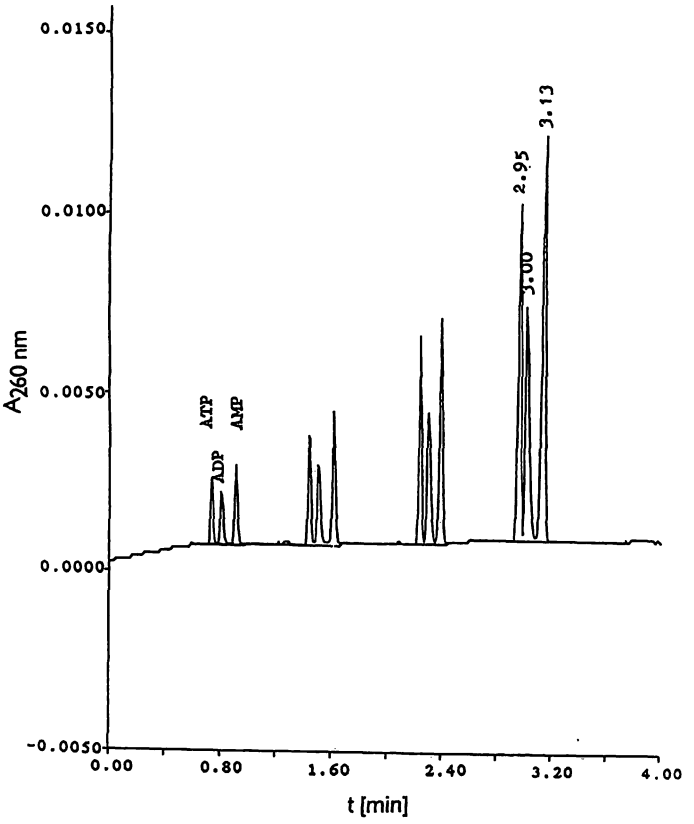
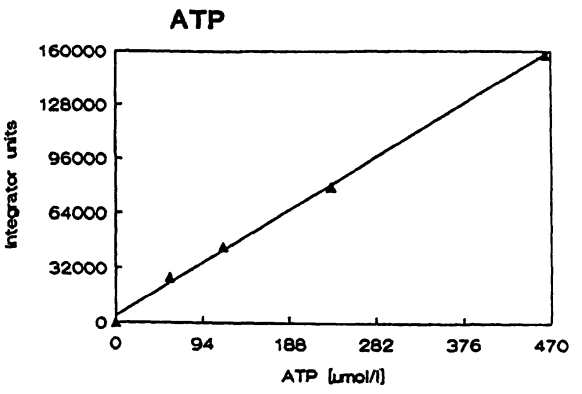
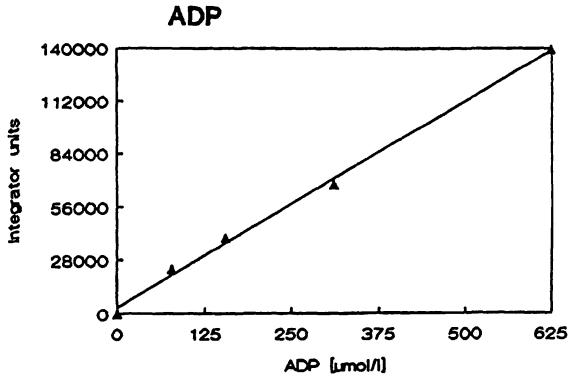


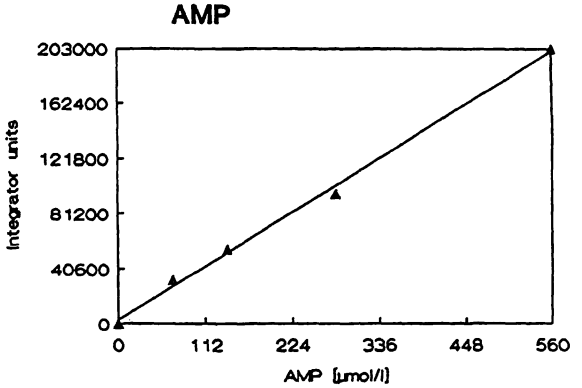
Fig. 2a The analyses of ATP, ADP and AMP standard solutions. Concentrations see figure 2b. Separation conditions: coated silica capillary (25 µm I.D. × 24 cm, 19.4 cm to the window). Operational borate buffer 0.23 mol/l, pH 7.8. Injection of sample 20 psi (14 × 10^3 kg/m^2) × s, 20 °C, 10 kV, polarity ⊖ → ⊕, detection wavelength 260 nm.



No.	ATP (µmol/l)	Integrator units
1	0	0
2	58	26393
3	116	44511
4	232	79900
5	464	157725



No.	ADP (µmol/l)	Integrator units
1	0	0
2	78	23075
3	156	39682
4	312	67711
5	624	139422



No.	AMP (µmol/l)	Integrator units
1	0	0
2	70	32297
3	140	54959
4	280	96046
5	560	202806

Fig. 2b Calibration graphs of ATP, ADP and AMP. Data from analyses see figure 2a.

Tab. 1 The migration times (mean ± SD) and apparent electrophoretic mobilities (μ_{app}) of adenosine phosphates standard solutions separated by capillary zone electrophoresis. For separation conditions and other data see figures 2a, 2b.

Nucleotides	Migration time (min, $\bar{x}$ ± SD)	μ_{app} 10^{-4} cm^2 V^{-1} s^{-1}
Adenosine 5'-triphosphate disodium salt 3 H_2O M_r 605.2, Boehringer Mannheim (58–464 µmol/l)	2.96 ± 0.11	2.62
Adenosine 5'-diphosphate sodium salt M_r 449.2, SIGMA (78–624 µmol/l)	3.02 ± 0.11	2.57
Adenosine 5'-monophosphate disodium salt 6 H_2O M_r 499.2, Boehringer Mannheim (70–560 µmol/l)	3.13 ± 0.12	2.47

Tab. 2 Migration times (mean ± SD, n = 7) reproducibility of migration times and peak area of nucleotides in whole blood. Separation conditions: coated silica capillary (24 cm × 25 µm, 19.4 cm to window), 0.23 mol/l borate buffer (pH 7.8), 20 °C, 20 kV, sample injection 20 psi (14 × 10^3 kg/m^2) × s, electrode polarity ⊖ → ⊕, detection wavelength 260 nm.

Nucleotide	Migration time (min, $\bar{x}$ ± SD)	Reproducibility of migration time (%)	Peak area CV (%)
ATP	1.57 ± 0.038	2.55	5.59
ADP	1.62 ± 0.041	2.53	5.32
AMP	1.67 ± 0.041	2.44	5.64

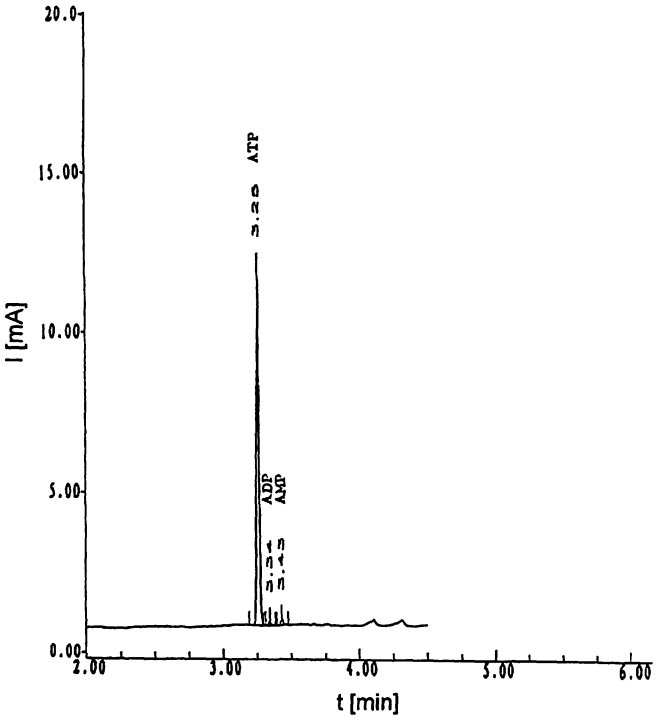


Fig. 3 The analysis of ATP in a mixture of ATP standard solution (428 µmol/l) and blood deproteinate with ATP concentration 393 µmol/l (1 : 1). The fusion of the analyte peak from blood deproteinate together with the pure ATP preparation peak gives evidence of identity of these components. For analysis conditions see figure 2a.

Tab. 3 Concentrations of nucleotides in whole cord blood from newborns immediately after birth. Clinical data of seven full-term babies without signs of perinatal asphyxia (mean ± SD) ♀ = 5, ♂ = 2.

Gestational age (weeks)	Birth mass (g)	Apgar score	ATP µmol/l	ADP µmol/l	AMP µmol/l
40.0 ± 1.1	3310 ± 292	1 min 7.4 ± 1.8 5 min 8.6 ± 1.4 10 min 9.3 ± 0.7	467 ± 134	68 ± 59	46 ± 14

tides examined can be confirmed by the addition of internal standards of relevant pure nucleotides to the blood deproteinates, resulting in fusion of both analytes in the single peak (fig. 3). The application of the method is illustrated by the determination of ATP, ADP and AMP (mean ± SD) in cord blood from seven full-term newborns delivered after normal pregnancies without signs of perinatal asphyxia (tab. 3). The nucleotide concentrations determined in whole blood agree with the findings of other workers (7). The method provides a detection limit for adenosine nucleotides of about 5 µmol/l. The results of two electrophoretic analyses from one physiologic newborn (fig. 4) and from another preterm newborn infant with perinatal asphyxia are demonstrated (fig. 5). The latter, with perinatal asphyxia, shows a corresponding significantly decreased ATP concentration. In spite of the ATP, ADP and AMP contents in blood, deproteinates from physiological newborns exhibit wide ranges, still the decreased ATP concentration serves as a useful indicator of threatening or persisting hypoxia.

Discussion

The occurrence of purine nucleotides in blood depends on the content in red cells and platelets, whereas plasma under normal conditions does not contain any of these compounds. According to our first experience the decrease of ATP during hypoxaemia does not correlate with the red cell and platelet count but rather with the extent of asphyxia. The earlier studies on the concentration of nucleotides in blood do not take into account the counts of red cells and platelets (7).

The analyses recorded in figures 1, 4 and 5 were performed at the voltage 20 kV, while those in figures 2a and 3 were run at voltage 10 kV. Higher voltages make it possible to shorten the time of analyses substantially even at the risk of shortening the life of the inner surface coating of the 25 µm inside diameter capillary.

Capillary electrophoresis allows simultaneous and specific determination of adenosine phosphates in whole blood with a single analysis, which could not be reached

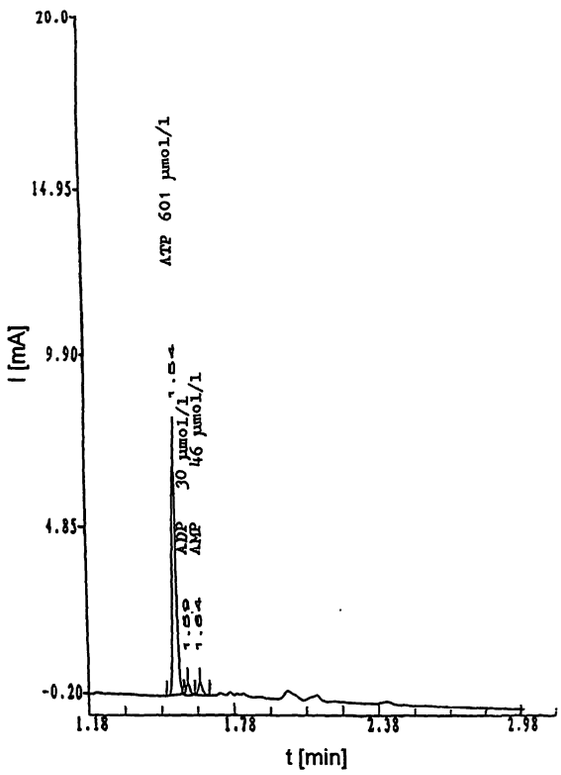


Fig. 4 The adenosine phosphates in cord blood, delivery of the full-term newborn in the 40th week of pregnancy, body mass 3300 g, transient hyperbilirubinaemia, phototherapy for 20 h, breastfed, discharged on the 5th day without complications. For separation conditions see figure 1a.

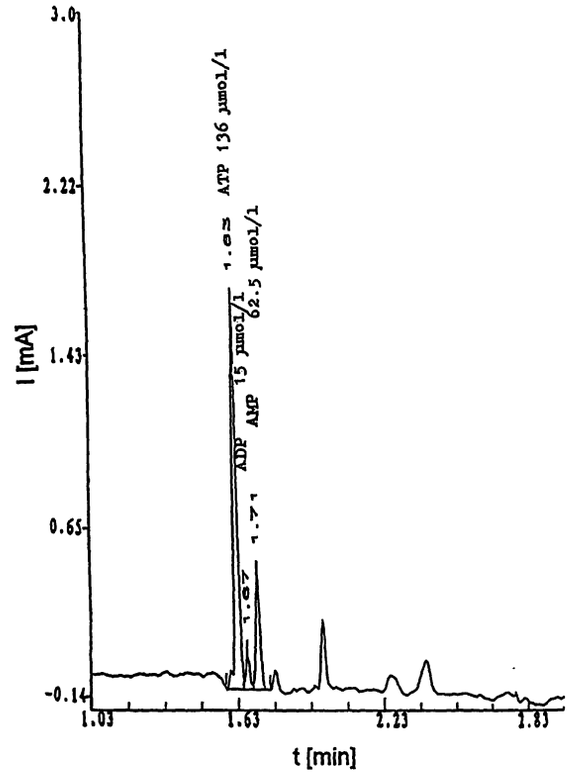


Fig. 5 The adenosine phosphates in cord blood, delivery of preterm baby in the 36th week of pregnancy. Body mass 2600 g, asphyxia, hypoxia intra partum, icterus neonatorum, oxygenotherapy for 24 h, hospitalized for 12 days. For separation conditions see figure 1a.

with either the enzyme or bioluminescent methods. It provides useful information about the pool of chemical energy within an organism and thus enables monitoring of unusual metabolic situations. Capillary electrophoresis represents a new potentially important separation technique because it brings speed, quantitation, reproducibility and automation to the inherently resolving technique of electrophoresis.

Acknowledgements

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References

1. Janssens D, Michiels C, Delane E, Eliaers F, Drieu K, Remacle J. Protection of hypoxia-induced ATP decrease in endothelial cells by Ginkgo biloba extract and bilobalide. Biochem Pharmacol 1995; 7:991–9.
2. Kamarýt J, Muchová M, Stejskal J. Determination of adenosine phosphates in whole blood by capillary zone electrophoresis. In: Martin SM, Halloran SP, editors. Proceedings of the XVI International Congress of Clinical Chemistry 1996 July 8–12, London 1996. Piggot Printers Limited, Cambridge 1996:428.
3. Grune T, Ross GA, Schmidt H, Siems W, Perrett D. Optimized separation of purine bases and nucleosides in human cord plasma by capillary zone electrophoresis. J Chromatogr 1993; 635:105–11.
4. Dawson JE, Nichols SC, Taylor GE. Determination of impurities in a novel analogue of adenosine-5'-triphosphate by capillary electrophoresis. J Chromatogr 1995; A700:163–72.
5. BioFocus Capillary Electrophoresis System. Instruction Manual, Version 5.00, BIO-RAD Laboratories, CA, USA 1995.
6. Landers JP. Handbook of Capillary Electrophoresis. Boca Raton, Ann Arbor, London, Tokyo: CRC Press 1994.
7. Methods for Clinical Chemical Research. Biochemica Boehringer Mannheim 1988/1989; 20–5.

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